THE PRESENCE AND SIGNIFICANCE OF RESPIRATORY METABOLISM IN STREAK-FORMING CHICK BLASTODERMS[1]

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The value of simple sugars, particularly glucose, in early chick development is well known (Needham and Nowinski, 1937; Spratt, 1949; Taylor and Schechtman, 1949; Fraser, 1954a). Needham (1931) and Romanoff and Romanoff (1949) list glucose as a free constituent of egg yolk, and recent investigation (Fraser, unpublished results) has shown that glucose is the only free monosaccharide in egg white dialysate detectable by chromatographic procedure. In 1938 Jacobson found that there was a marked glycolysis in involuting mesodermal cells at gastrulation. Such studies have illustrated the importance of carbohydrate metabolism in early chick embryogenesis.

While the importance of carbohydrate utilization is generally recognized, the manner in which it is metabolized has been disputed. Novikoff, Potter and LePage (1948) stand against the contention of Needham and Nowinski (1937) that there is a non-phosphorylating glycolytic scheme in young chick blastoderms. The former authors were able to detect assorted phosphorylated carbohydrate components in embryos of three to ten days incubation. Needham and Nowinski were unable to find an increase in oxygen consumption either in whole embryos or homogenate, when phosphorylated sugars were added.

Aside from the energetics involved at this level of glycolysis, terminal oxidation with molecular oxygen by cytochrome oxidase has been followed. Using manometric means, Potter and DuBois (1942) found the first evidence of this enzyme in the six-day embryo. Albaum and Worley (1942) were able to detect activity, as measured by oxygen uptake, in the embryo of four days. By soaking blastoderms in solutions containing dimethyl-p-phenylenediamine and alpha naphthol (nadi reagent), followed by visual inspection, Moog (1943) has been able to show that cytochrome oxidase is present even in head process stages. Sodium azide-treated embryos lost much of the respiratory activity seen in the experimental group.

Moog also found that this enzyme activity was expressed in a morphological pattern similar to that displayed by reducing enzymes (Spratt, 1951a), sensitivity to respiratory poisons (Hyman, 1927; Spratt, 1950b), anaerobiosis (Spratt, 1950a) and starvation (Spratt, 1951b; Fraser, 1954a). In general, these experiments have revealed that the node and fore-brain are regions of high metabolic activity. I (Fraser, 1954a) have been able to demonstrate that the node is very susceptible to degenerative changes on starvation, while the brain is relatively refractory to such treatment.

It is the purpose of the present paper to determine if there is indophenol oxidase

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(cytochrome oxidase according to Keilen and Hartree, 1938) activity in earlier, streak-forming stages of the chick, and if so, if there is some pattern in its distribution. Further, if certain cells do show a greater respiratory metabolism, another objective in mind is to investigate the possibility that it has some significance in the differentiation of these cells.

The results from the present work permit the following statements. Cytochrome oxidase is detectable in streak-forming chick blastoderms, particularly in the newly involuted mesoderm cells. There is no change in the activity of this enzyme in embryos on the nadi reagent following pretreatment with cytochrome *c* or albumen, but it increases appreciably in blastoderms starved for five hours in saline. There is a striking decrease in its activity, as measured by indophenol formation, in explants treated mildly with hydroquinone, a reductant presumably for cytochrome *c*. The augmented respiratory metabolism seen in newly formed mesoderm cells is related in some manner with differentiative ability at the trunk level of the chick blastoderm, since fragments of involuted mesoderm expressed ability to form mesoderm tissue, while potential mesoderm fragments (axial epiblast in broad- and intermediate-streak embryos) failed in this respect.

Experimental Procedures and Results

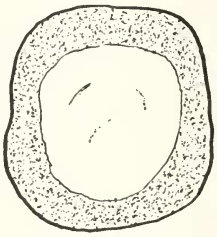
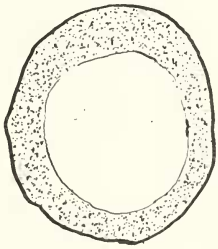
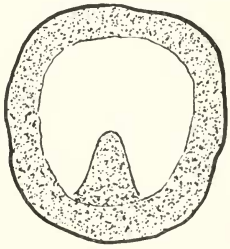
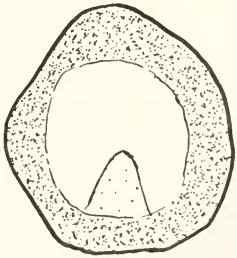
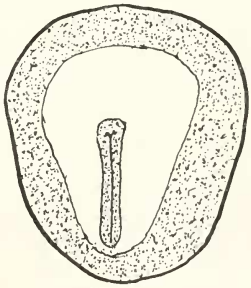
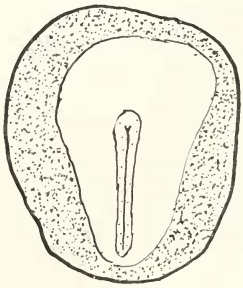
Newly laid fertile eggs were obtained from the poultry farm of the University of Tennessee. Most of the eggs used were from White Leghorn chickens, although a few were from Rhode Island Red stock. They were stored in the refrigerator at 18° C. upon receipt until the time (within five days) they were used in the experiments. The procedure for removing blastoderms from the yolk and for explanting them in culture has been given previously by Spratt (1947). Eggs were incubated in a forced draft incubator at 37.8° C., while explants were cultured at 37.6° C. The temperature in both incubators was maintained at a constant value by mercury type thermoregulators. Manipulation of the blastoderms was carried out under Ringer's solution. Specimens for histological inspection were fixed in Bouin's fluid and stained with Delafield's hematoxylin. Over six hundred blastoderms were used in the course of this investigation.

A. Aerobic enzyme pattern in early-streak blastoderms

Pre-, intermediate- and definitive-streak (DPS) embryos corresponding to stages 1, 2–3 and 4 of Hamburger and Hamilton (1951), respectively, were removed from the yolks under saline, and explanted onto freshly prepared nadi reagent, made in the following manner. Five ml. of 0.08 *M* dimethyl-p-phenylenediamine in chick Ringer's, 5 ml. of 0.08 *M* alpha naphthol in 20% ethyl alcohol-Ringer's, 1 ml. of bicarbonate buffer and 2 ml. of phosphate buffer were added to 27 ml. of Ringer's containing 300 mg. agar, after the saline-agar had been heated and then cooled to approximately 50° C. In every experiment outlined here and subsequently in which the nadi reagent was used, fresh preparations of dimethyl-p-phenylenediamine and alpha naphthol were made up immediately before use, because the former compound is oxidized rather rapidly by atmospheric oxygen. Preparation of the buffers used has been described previously (Fraser, 1954a). At this time, however, saturation of the bicarbonate buffer with CO_2 was achieved by passing the gas from a tank through a nozzle into the solution. The final pH of the 0.01 *M* nadi reagent

TABLE I

Pattern of cytochrome oxidase activity in early chick embryos explanted onto media containing dimethyl-p-phenylenediamine and alpha naphthol (nadi reagent) and nadi reagent with sodium azide

NO.	GENERALIZED RESULTS	
	NADI REAGENT (.01 M)	NADI REAGENT (.01 M) Na AZIDE (.005 M)
36		
34		
38		

medium was 7.1. The preparation was poured immediately into watch glasses held in petri dishes by moisture-saturated cotton rings. Gelation of the medium occurred within a few minutes. A control medium containing sodium azide at a final concentration of 0.005 *M* was made up in a similar manner.

The embryos were explanted onto the nadi reagent or nadi reagent-azide media and incubated for fifteen minutes at 37.6° C. At precisely this time they were removed from the media by pipette and examined under saline against a white background through a dissecting microscope.

The generalized observations are shown in Table I. Cells in the opaque area peripherad to the germ wall in all three stages tested showed a dark blue-purple coloration on the nadi reagent. No other pattern of coloration indicative of cellular respiration could be found in pre-streak embryos. A dark color was apparent, however, in the streak-forming region of the intermediate-streak blastoderm and along the streak and node area in the DPS explants. Other pellucid area tissues were very faintly stained. On the azide-bearing medium, the yolk-laden cells in the area opaca retained, in large part, a deep coloration, while streak tissues were essentially colorless. In fact, it was extremely difficult to see the developing or full streak against the white background in embryos removed from the medium containing the azide.

The logical interpretation to be made from the above results is that the coloration expressed in the vitelline cells surrounding the embryo is due mainly to a non-enzymatic mechanism. Since interest was directed toward a localization of activity in the embryo proper, this issue was not pressed, although it may be conjectured that the amount of tissue and yolk in this region may be sufficient to soak up enough indophenol from the surrounding fluid to give this appearance. Other interpretations may be advanced, but failure of the azide to prevent coloration must mean that known oxidative enzymes are not involved. Furthermore, the marked depression in indophenol formation in streak tissues on the azide medium must indicate that this coloration is mediated by enzyme action. There is good evidence that azide inhibits the action of indophenol oxidase (cytochrome oxidase) which is directly responsible for the formation of the bluish-purple indophenol (Keilin, 1936) as well as transphosphorylation and ATPase activity (Meyerhoff, 1945).

To insure that the darker color in the region of the forming and full streak was not due simply to the presence of more cells compacted at this region, fragments of tissue of comparable thickness were removed from streak epiblast, mesoderm and hypoblast for inspection. For comparison, fragments of non-axial epiblast were also examined. These pieces were placed side by side on a microscope slide in a small amount of fluid and covered with a cover slip. The stained tissues prepared in this manner were examined for intracellular indophenol deposition.

Figures 2 and 3 will reveal that enzymatic activity is greater in involuted mesodermal cells than in overlying epiblast (potential mesoderm in intermediate-streak embryos) cells. Attention is drawn to the fact that indophenol is produced at the surface of small droplets in the cells. The cytoplasm of the cells is relatively free from coloration. This is typical of all cells observed. It may also be seen that while the number of droplets is essentially the same in both epiblast and mesoderm cells, the enzymatic activity is greater on the surface of those in newly involuted cells. These globules are readily stained with Sudan III, indicative of a lipid con-

tent. These photographs are of living cells removed from a streak-forming blastoderm. In obtaining the photographs care was taken to make sure that identical conditions, such as illumination, exposure time, time of processing, etc., were maintained. The similar appearance in the photographs of a defect in the lens of the photographic equipment will attest this. Such inspection revealed that as far as indophenol oxidase activity is concerned, streak hypoblast and all epiblast cells tested were the same. It is clear that the darker coloration of the region of the forming streak or in the node and full streak of the older blastoderms is due solely to a greater respiratory activity in newly involuted mesoderm cells. This observation is in conformity with that of glycogen utilization by invaginating mesoderm made by Jacobson (1938).

B. *Modification of cytochrome oxidase activity by pretreatment*

We are dealing here with an enzyme which has as its substrate the nadi reagent under experimental conditions and presumably cytochrome *c* in normal cellular respiration. Therefore, on theoretical grounds at least, it should be possible to modify the reaction between the enzyme and the nadi reagent by the addition of the normal substrate. If living cells behave as does mammalian heart muscle extract, according to the observations of Keilen and Hartree (1938), we should expect an increase in oxidation of the diamine on addition of cytochrome. At the same time, a depletion of readily metabolizable food reserves in the cell, resulting in a depressed enzyme activity, could also conceivably lead to greater nadi oxidation, and hence augmented coloration. These ideas were followed by the following experimentation. Four dozen eggs were supported on their sides in a tray and left in the refrigerator at 18° C. overnight, so that the position of the blastoderm would be known. On the following day 0.2 ml. of 1.4×10^{-4} *M* cytochrome *c* was injected into the yolk sacs of two dozen eggs, while a similar quantity of 2% sodium succinate was injected into the other two dozen. Based on previous measurements of frozen eggs a needle of sufficient length was chosen so that the injected materials would be placed about one quarter inch from the blastoderm. The needle was inserted vertically from the lower side of the egg to avoid possible injury to the blastoderm. Both preparations that were injected were sterilized by filtration. The concentration of the cytochrome *c*, prepared according to Umbreit *et al.* (1949), was established by use of the Beckman spectrophotometer. The eggs thus treated were incubated for ten hours after which they were explanted onto the Nadi reagent in the manner described above.

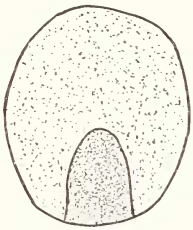
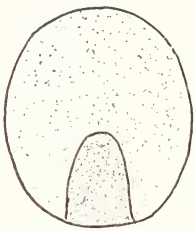
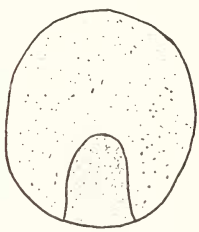
The results were rather disappointing in that there was no difference in enzymatic activity in either of these groups of embryos as compared to normal, non-injected controls. It became clear that the question as to whether the negative results were due to the inactivity of the materials on the blastoderms or to the failure of the materials to reach the embryos could not be resolved by this procedure, so it was abandoned.

Next, early streak blastoderms were removed from the yolk in the usual manner after ten hours of incubation, and incubated in various liquid preparations. These included: (1) saline, (2) albumen, (3) albumen-cytochrome *c*, (4) albumen-cytochrome *c*-hydroquinone and (5) albumen-hydroquinone. The final concentration of cytochrome *c* was $1.4 \times 10^{-5}M$, that of hydroquinone $10^{-3}M$. The albumen con-

centration was the same as previously employed in agar gels. Pretreatment time for those in the first three media indicated was five hours. It has been shown (Spratt, 1951b; Fraser, 1954a) that permanent damage occurs in embryos explanted on non-nutrient media for intervals longer than this. Blastoderms were pretreated in the media containing hydroquinone for one hour, since beyond this time cell dispersal began. Twenty-four embryos were placed in each medium, incubated for the period indicated above, removed, washed thoroughly in saline and immediately explanted onto a nadi reagent-bearing agar medium. On this they were again incubated for fifteen minutes after which they were removed, placed in Ringer's and inspected. Embryos of the same age were removed directly from eggs and treated on the nadi reagent for the same length of time for comparison.

TABLE II

Relative expression of cytochrome oxidase activity in streak-forming chick blastoderms pretreated with materials indicated

	GENERALIZED RESULTS		
PRETREATMENT MEDIUM	SALINE	ALBUMEN ALBUMEN–CYTOCHROME C CONTROL	ALB.–CYT.C–HYDROQUINONE ALBUMEN–HYDROQUINONE
			
NUMBER	24	60	48

The results are shown in generalized form in Table II. To avoid confusion, the three groups of media producing different results will be treated separately.

(1) *Albumen, albumen-cytochrome c, control (no previous treatment)*: the sixty blastoderms in this group were all similar to those described previously with respect to indophenol oxidase pattern and intensity of color. It appears obvious that neither the albumen nor the cytochrome *c* had any affect on the enzyme activity when presented to the embryos in this manner.

(2) *Saline (non-nutrient)*: Blastoderms incubated in this fluid stained most intensely by nadi reagent. All embryonic tissues were slightly darker than those on media listed above, but streak tissue was considerably more colored. A comparison of cellular details in fragments of tissues from streak epiblast, mesoderm and hypoblast between these and albumen-treated embryos revealed that coloration was darker in all three germ layers in prestarved embryos, with newly involuted mesoderm again showing the greatest indophenol deposition (Fig. 4).

(3) *Albumen-cytochrome c-hydroquinone, albumen-hydroquinone:* After pretreatment in these media, blastoderms showed a striking decrease in indophenol coloration when incubated on the nadi reagent. All tissues seemed to be stained somewhat less, but again the streak-forming tissues seemed most influenced by pretreatment. Although not nearly as faintly colored as those on an azide medium, these tissues nevertheless were considerably lighter in appearance than in control blastoderms.

In all cells observed, the blue color was localized on the surface of intracellular globules, even in starved embryos. It is interesting to note that cells showing the greatest enzyme activity are those of starving blastoderm mesoderm, and that this activity is on the surface of lipid material. The significance of this and of other observations made at this time will be discussed more fully later. It will suffice to point out here that the less intense color on the droplets in cells of hydroquinone-treated animals represents a decrease in dimethyl-p-phenylenediamine-alpha naphthol oxidation. This is what one would expect if one assumed that the hydroquinone acts specifically as a reductant (as has been shown by Krahl and co-workers, 1941, in sea urchin eggs) for intracellular cytochrome *c* and not indophenol, and provided that the cells were not killed by such treatment.

Considering the first assumption, it was determined that hydroquinone, in the concentration used in the experiment, and even in much greater concentration, could neither prevent the formation of indophenol from fresh nadi reagent, nor could it reduce indophenol to the leuco form *in vitro*. Secondly, other blastoderms treated with hydroquinone as indicated were subsequently washed thoroughly and explanted onto an albumen-agar medium. These were then incubated for twenty-four hours. Although development did not proceed as in normal explants, there was some slight morphogenesis, and tissues did not have an opaque appearance characteristic of death of the cells.

C. *Non-autonomy of the increase in cytochrome oxidase activity*

The question arose as to whether the increase in enzymatic activity seen in involuted mesoderm was a function of time or of location of tissue. Preceding statements have indicated that after a pretreatment interval of five hours, there was less indophenol localized in epiblast cells than in mesodermal cells. But coincident with change in time there has been some involution during pretreatment.

Small fragments of tissue taken from streak epiblast and streak mesoderm were removed from streak-forming blastoderms and cultured under albumen (prepared as previously outlined) for intervals from five to ten hours. These were then washed in saline and explanted onto the nadi reagent medium for fifteen minutes and inspected. Similar fragments removed from blastoderms of the same age, but removed directly from eggs, were stained as controls.

The cultured streak epiblast tissue had the same blue indophenol coloration as the controls. Streak mesoderm cells from both groups also looked identical, although darker in appearance than epiblast cells.

By preventing involution in this manner in epiblast tissue (prospective mesoderm), cultured for a sufficient period of time for this basic morphogenetic phenomenon to have occurred, it was thus possible to show that increase in indophenol oxidase activity is not autonomous in this tissue. It seems clear that the gain in

respiratory activity is either due to movement of cells through the streak in gastrulation or to the influence of surrounding cells in a new mesodermal location. The following experiments are directed toward this question.

D. *Significance of increased respiratory activity of cells in histogenesis*

If an increase in enzyme activity in cells is due simply to placement of the cells, it should conceivably occur in streak epiblast cells implanted in a mesodermal location in early chick embryos. Eighteen fragments of such tissue were implanted through small tears in the hypoblast into positions indicated in Figure 1. Fragments were removed from stage 2 (Hamburger and Hamilton, 1951) embryo streak epiblast, while hosts were of stages 2 and 4. Blastoderms with implanted tissue were then incubated for six hours on a regular albumen-agar medium, after which

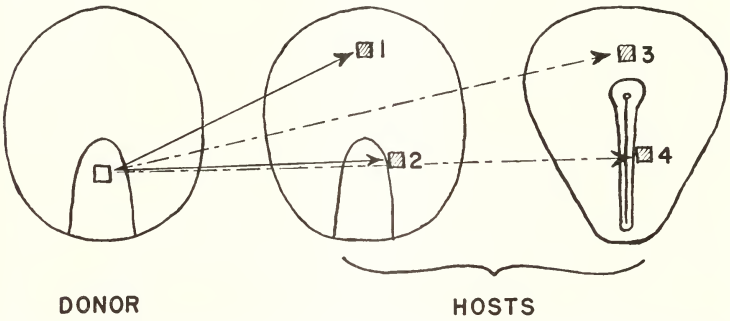


FIGURE 1. Illustration of sites for implanting fragments of streak epiblast into mesodermal locations of intermediate-streak and DPS blastoderms.

they were removed and explanted onto the nadi medium and reincubated for fifteen minutes. The implants, when found, were then removed under saline and mounted on microscope slides along with nadi-stained fragments of epiblast removed directly from streak-forming embryos for comparison. About one-third of the implants were extruded during incubation on albumen and were hence lost.

Fragments placed at mesodermal positions 2 and 4 in Figure 1 looked identical to control pieces. There had been no increase in enzyme activity in cells placed in these positions. From this and the foregoing observation, we might conclude that the increase in cytochrome oxidase activity in newly involuted cells is neither a direct function of time nor location, but is tied in with the morphogenic movement of the cells through the streak. This at least seems true for cells at the level of the streak in the early chick blastoderm.

It was rather surprising to see that implants into mesoderm in the prospective head region (positions 1 and 3 in Figure 1) were considerably darker than the controls. On the cell level, there was perceptibly more indophenol on the globules in cells of the implants than of the control fragments. At this region of the embryo, it appears that the development of intracellular catalytic activity is related to location of the cells.

These experiments were then followed by others to test the significance of this increase in observed enzymatic activity with respect to differentiative ability of the tissues involved. Fragments of streak epiblast were excised from streak-forming embryos and marked very lightly with diluted India ink under saline. They were then placed in saline and larger particles of carbon were removed with a steel needle. After a final washing, the pieces of tissue were implanted into mesodermal sites indicated in Figure 1, in both streak-forming and DPS hosts. Implantation occurred through small tears made in the hypoblast at the desired regions. In a similar manner, small pieces of newly involuted mesoderm from the same embryos were implanted in the same regions with the exception of the head region in early streak embryos. The host blastoderms were then cultured on albumen-agar for twenty-four hours at 36.7° C., fixed with Bouin's fluid and prepared for paraffin impregnation. The serial sections were made 12 microns in thickness. Eighteen implants were made into each site indicated.

TABLE III

Summary of results from implanting fragments of streak epiblast of streak-forming (SF) chick embryos into mesodermal sites of host embryos.

Type of fragment	Site of implantation	Fragments recovered*	Results
Epiblast	Head SF	12	Head mesoderm and pharynx
Epiblast	Head DPS	9	Head mesoderm and pharynx
Epiblast	Flank SF	15	Isolated ball degenerative cells
Epiblast	Flank DPS	13	Isolated ball degenerative cells
Streak meso.	Head DPS	12	Head mesoderm and pharynx
Streak meso.	Flank SF	10	Ball of living cells Flank mesoderm
Streak meso.	Flank DPS	10	Ball of living cells Flank mesoderm

* Number of blastoderms showing some evidence that tissue had been implanted.

The results are given in Table III. It will become immediately evident that there is a good correlation between increased cytochrome oxidase activity and differentiative ability. Fragments of epiblast placed in prospective head mesoderm not only show an increase in metabolic (oxidative) activity but also display an expanded capacity for differentiation. Implants in flank regions failed to show any increase in enzymatic activity coincident with a failure to produce mesodermal structures. It is also apparent that involuted mesoderm has the capacity to form both mesoderm and endoderm in the head region as well as flank mesenchyme. There are limitations to this ability at the trunk level, however. Figures 5–10 are photographs illustrating the results obtained. Engulfed particles located in head mesoderm and pharynx are taken as evidence that these cells had differentiated from implanted tissue.

DISCUSSION

The work of Moog (1943) has established that cytochrome oxidase is present in chick embryos in stages as early as those possessing a head process. In view of the fundamental importance of this enzyme in many diverse organisms, there

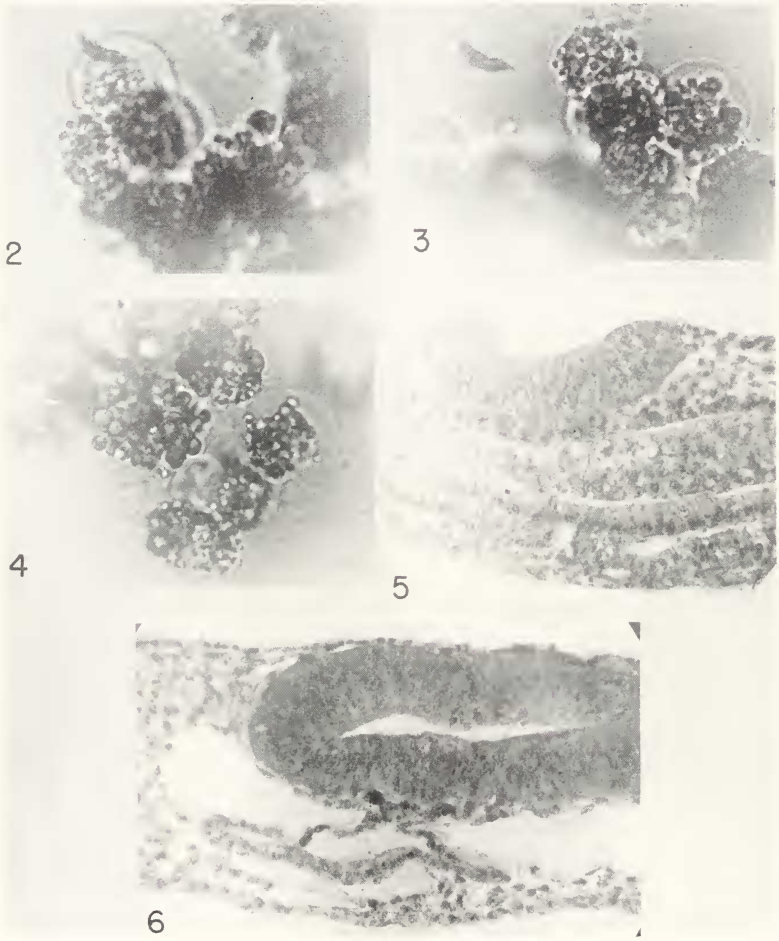


FIGURE 2. Living nadi reagent-treated cells from streak epiblast of an intermediate-streak blastoderm. Note that indophenol is deposited on the surface of intracellular droplets. × 960.

FIGURE 3. Photomicrograph of living cells stained on the nadi reagent. These cells are from newly involuted mesoderm of an intermediate-streak embryo. × 960.

FIGURE 4. Stained streak mesoderm cells from a pre-starved intermediate-streak blastoderm. × 960.

FIGURE 5. Carbon-marked cells in pharyngeal endoderm of a stage 2 explant after twenty-four hours of subcultivation. The brain at this level has not rolled completely into a tube. × 120.

FIGURE 6. Carbon engulfed by mesoderm and pharynx of a DPS explant after one day of subcultivation. ×120.

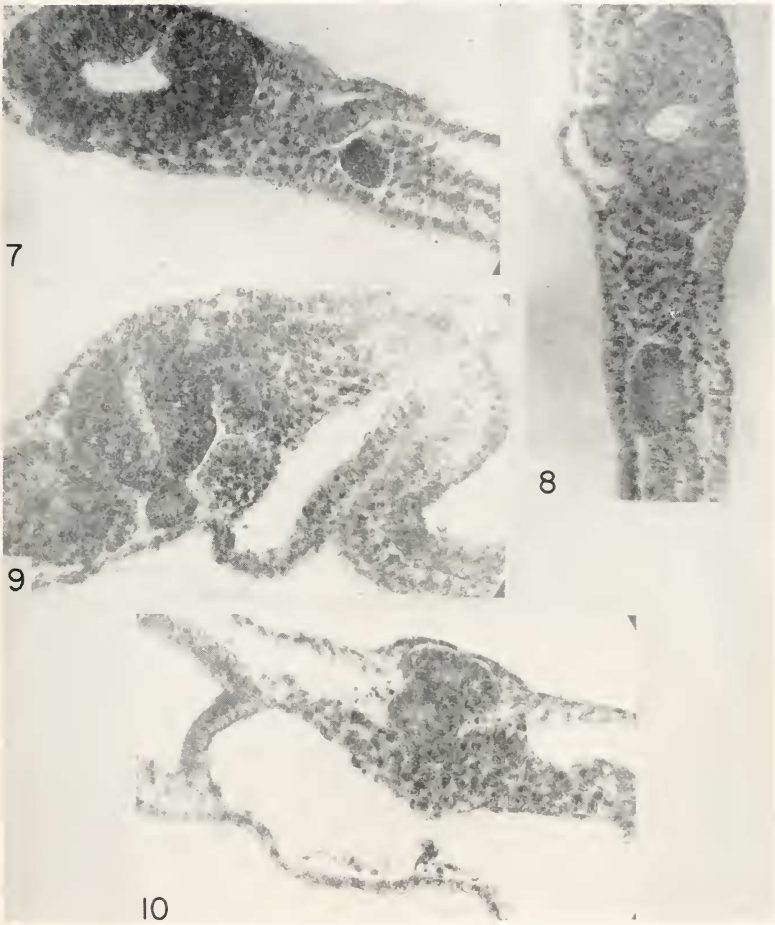


FIGURE 7. Remnant of an epiblast implant grafted into flank mesoderm of a DPS host followed by twenty-four hours of incubation. ×120.

FIGURE 8. Photomicrograph of an isolated ball of degenerative epiblast cells in flank mesoderm of a stage 2 blastoderm host after one day of subcultivation. × 120.

FIGURE 9. Isolated mass of viable cells in mesoderm initially implanted in the flank region of a DPS host. Photograph taken twenty-four hours postoperatively. Donor cells were from newly involuted mesoderm of a stage 2 blastoderm. × 120.

FIGURE 10. Photomicrograph of carbon-marked flank mesoderm cells in a stage 2 host following one day of subcultivation. Donor tissue was newly involuted mesoderm from a stage 2 embryo. × 120.

is no reason to believe that it may not be present in even earlier chick blastoderms. It has been detected by Krahl and co-workers (1941) in pre- and post-fertilized Arbacia eggs. Regardless of the type of food being utilized, as long as aerobic oxidation occurs it seems likely that this terminal enzyme will be involved. It is common knowledge that the chick embryo uses oxygen continuously following laying of the egg. The results of the present investigation have shown that cytochrome oxidase is indeed present in very early embryos, detectable by the nadi reagent under the conditions utilized even in streak-forming stages. Before this time it is probable that this enzyme is present but is not specifically outstandingly active at any localized region.

The specific significance of the observed rise in activity of cytochrome oxidase in involuting cells can only be conjectured. Certainly it is associated in some manner with the differentiative process, since it is accompanied by an expansion of possible fates in cells in which it occurs. It may well be that the increased energy liberated in these cells at this time may be directed toward anabolic processes. That differentiation is accompanied by aerobic oxidation has very recently been pointed out by Warburg (1956). The correlation between oxidative activity and histogenesis appears of prime significance.

Previously (Waddington, 1932; Fraser, 1954b) it has been shown that an interchange of cells between head endoderm (pharynx) and head mesenchyme is much in evidence in early chick embryos. It may well be that tissues in this region are more influenced in their differentiation by cells about them than they are at lower (trunk) levels. No evidence has been found in the present investigation to support the observations of Waddington and Taylor (1937) that epiblast tissue implanted at lower regions of the chick blastoderms would form mesodermal structures.

Mention should be made of the results obtained in experiments dealing with nadi oxidation in blastoderms pretreated with various materials. If we assume that there is competition between the nadi reagent and cytochrome *c* for the enzyme cytochrome oxidase, then certain interpretations of the results can fruitfully be made. This assumption is in sharp contrast to the ideas of Keilen and Hartree (1938), but see below. It is well known that both of these materials are oxidized by this enzyme in the presence of molecular oxygen to indophenol and oxidized cytochrome *c*, respectively. If we accept the assumption of a competition of substrates, the increase in nadi oxidation in starved embryos could mean that normal oxidation through cytochrome *c* is curtailed, presumably due to exhaustion of utilizable carbohydrate reserves (free hexoses). Dimethyl-p-phylenediamine-alpha naphthol would therefore be oxidized more readily, leading to the more pronounced coloration observed.

Indophenol intensity was the same in embryos pretreated with albumen-cytochrome *c* as in controls. At the same time, embryos incubated in albumen-cytochrome *c*-hydroquinone and albumen-hydroquinone were perceptibly less colored. If cytochrome *c* could enter the cells, we should expect a decrease in nadi oxidation. The fact that hydroquinone in the absence of cytochrome *c* produced the same result as with it suggests that the cytochrome is not gaining entrance to the cells. Krahl *et al.* (1941) and Keilen and Hartree (1938) using preparations of Arbacia eggs and mammalian heart muscle, respectively, have shown that there is an increase in cytochrome oxidase activity proportional to the amount of cytochrome *c* added. The size of the cytochrome (molecular weight of 13,000 according to

Potter, 1950) should not be a serious detriment to cell entry, since larger compounds are suspected of entering cells. Nevertheless, the results would indicate that it did not enter the cells. It is obvious, however, that the hydroquinone had. In view of the fact that this material will not reduce indophenol, but has been shown to reduce cytochrome *c* (Krahl *et al.,* 1941) one is led to the conclusion that the hydroquinone selectively and persistently reduces the normally present intracellular cytochrome *c*, and hence the affinity for the oxidase with the nadi reagent is reduced.

There is, however, an alternative explanation which is more in keeping with the conclusions of Keilen and Hartree (1938). These workers found that the catalytic action of cytochrome oxidase on p-phenylenediamine was greatly enhanced by the addition of cytochrome *c*, and that this aromatic amine was oxidized much more readily by the enzyme than were other compounds, including hydroquinone. They state further that the rate of catalytic hydroquinone oxidation may be increased 30- to 40-fold on the addition of cytochrome *c* (10^{-5} to 10^{-4} *M*) to the preparation. Thus, rather than there being a competition between cytochrome *c* and hydroquinone for the enzyme, in heart muscle preparations at least, there is a dependency on the presence of the cytochrome for the catalytic oxidation of hydroquinone. Assuming this and again considering only the hydroquinone as entering the cell, it must be that intracellularly the hydroquinone has more affinity for the enzyme than has the nadi reagent. This is, of course, somewhat at variance with the English workers' observations. It may be that the difference in results lies in the materials and methods used. Intracellularly, structure may provide results differing from those obtained *in vitro*. At any rate, this interpretation is also in keeping with observations made.

Finally, consideration should be given to the perplexing problem of starvation of the chick embryos whose cells are amply supplied with high energy food material. Reference has already been made to the fact that chick blastoderms soon die when explanted on non-nutrient media. Spratt (1951b) has shown that recovery is possible in embryos starved for six hours on a saline-agar medium, when they are returned to an albumen substrate. I have found (Fraser, 1954a) that certain degenerative features are obvious in explants starved for ten hours. In checking recently, I have found that in embryos starved for ten hours, there are still many intracellular Sudan III-stainable globules. It thus becomes evident that lipids are not utilized, at least to any appreciable degree, by early chick blastoderms. This conclusion had been drawn previously by Needham (1931). This author, using R.Q. determinations as a basis, stated that during chick embryogeny carbohydrates are utilized for the first seven days of incubation (R.Q. = 1), proteins are used next and finally lipids are used only near the time of hatching.

Summary

1. Cytochrome oxidase has been detected in chick blastoderms as early as the intermediate-streak stage, by use of the explanting procedure on an agar medium containing dimethyl-p-phenylenediamine-alpha naphthol (nadi reagent). Intracellular indophenol deposition was localized on the surface of lipid droplets, particularly in newly involuted mesodermal cells. Enzymatic activity was negligible in embryos explanted on a similar medium containing sodium azide.

2. Nadi oxidation was augmented, notably in streak mesoderm of early explants after such blastoderms had been starved in saline for a period of five hours. Embryos pretreated in albumen-saline, or albumen-saline-cytochrome *c* for a similar interval showed no increase or decrease in intracellular enzymatic activity as compared to controls, when they were subsequently explanted onto the nadi-bearing medium. However, diamine oxidation in blastoderms treated in solutions containing albumen-cytochrome *c*-hydroquinone and albumen-hydroquinone was perceptibly decreased.

3. The development of the ability to oxidize the nadi reagent was not autonomous in fragments of streak epiblast (prospective mesoderm), but required normal involution at gastrulation. This was shown by pieces of this tissue implanted into trunk-level mesoderm. When implanted in a future head mesoderm location, however, such fragments did reveal an increase in enzymatic activity. When incubated in albumen-saline for intervals of time up to ten hours, small pieces of epiblast did not show an increase in nadi oxidation.

4. These results were correlated with the ability of the tissue fragments to form mesodermal and endodermal structures. Implants of epiblast placed in prospective head mesoderm of streak-forming and definitive primitive streak hosts were incorporated into head mesenchyme and pharyngeal tissue. Similar tissue when placed with other mesoderm at trunk levels failed to differentiate into mesenchyme. Newly involuted mesoderm from streak-forming blastoderms had the same fate as did epiblast fragments, when implanted in a future head mesoderm location. At the trunk level this tissue became integrated into mesoderm cells about it or formed semi-isolated balls of living tissue.

5. The significance of the observations, with respect to nutritional requirements of early chick blastoderms and the relationship between oxygen utilization and differentiation, is discussed briefly.

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